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A CONJUGATE BETWEEN A MODIFIED SUPERANTIGEN AND A TARGET-SEEKING
COMPOUND AND THE USE OF THE CONJUGATE.

Superantigens are primarily proteins of viral or bacterial
5 origin and are capable of simultaneous binding to MHC class II
antigens on mammalian cells and the T cell receptor V β chain. The
binding leads to activation of T-lymphocytes and lysis of the MHC
class II bearing cells. The moderate degree of polymorphism of
the binding part of the V β chain causes a relatively large
10 portion of the T-lymphocytes to be activated when contacted with
a superantigen (in comparison with activation through normal
antigen-processing).

Initially the superantigen concept was associated with various
staphylococcal enterotoxins (SEA, SEB, SEC₁, SEC₂, SED, and SEE).
15 Recently a new staphylococcal enterotoxin named SEH has been
discovered (Keyong et al., J. Exp. Med. 180 (1994) 1675-1683).
After the interest had been raised, further superantigens were
discovered. Examples are Toxic Shock Syndrome Toxin 1 (TSST-1),
Exfoliating Toxins (Exft) that are associated with scalded skin
20 syndrome, Streptococcal Pyrogenic Exotoxin A, B and C (SPE A, B,
and C), Mouse Mammary Tumor Virus Proteins (MMTV), Streptococcal
M Proteins, Clostridial perfringens enterotoxin (CPET) among
others. For a review of superantigens and their properties see
Kotzin et al. (Adv. Immunol. 54 (1993) 99-166).

25 Pseudomonas exotoxin A has been looked upon as a functional
superantigen because there are results indicating that this toxin
may be processed intracellularly by accessory cells to fragments
that are expressed on the cell surface with the ability to bind
to the V β chain and a subsequent activation of T cells.
30 (Pseudomonas exotoxin A. Legaard et al., Cell. Immunol. 135
(1991) 372-382).

Superantigens as such have been suggested for therapy of
various diseases with curative effects being accomplished through
a general activation of the immune system (Kalland et al., WO
35 9104053; Terman et al., WO 9110680; Terman et al., WO 9324136;
Newell et al., Proc. Natl. Acad. Sci. USA 88 (1991) 1074-1078).

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In connection with vaccines it has been suggested to use superantigens that have been mutated so as to lose their TCR binding ability (Kappler & Marrack, WO 9314634).

The mutation of superantigens has previously been described
5 (Kappler & Marrack, WO 9314634; Kappler et al., J. Exp. Med. 175 (1992) 387-396; Grossman et al., J. Immunol. 147 (1991) 3274-3281; Hufnagle et al., Infect. Immun. 59 (1991) 2126-2134).

We ourselves have previously suggested to employ conjugates between a superantigen and an antibody for therapy in order to
10 lyse cells that express the structure towards which the antibody is directed (Dohlsten et al., WO 9201470; Lando et al., Cancer Immunol. Immunother. 36 (1993) 223-228; Kalland et al., Med. Oncol. Tumor Pharmacother. 10 (1993) 37-47; Lando et al., J. Immunol. 150 (8 part 2) (1993) 114A (Joint Meeting of the
15 American Association of Immunologists and the Clinical Immunology Society, Denver, Colorado, USA, May 21-25 (1993)); Lando et al., Proc. Am. Assoc. Cancer Res. Annu. Meet. 33(0) (1992) 339 (Annual meeting of the American Association for Cancer Research, San Diego, California, USA, May 20-23 (1992)); Dohlsten et al., Proc.
20 Natl. Acad. Sci. USA 88 (1991) 9287-9291). Diseases suggested to be treated have been cancers, viral infections, parasitic infestations, autoimmune diseases and other diseases associated with cells expressing disease-specific surface structures. The experimental work carried out so far has focused on conjugates
25 containing recombinant SEA and various anti-cancer antibodies. The conjugates as such have had a somewhat reduced ability to bind MHC class II antigens compared to the non-conjugated form of the superantigen. It has not been determined if a decreased MHC class II antigen binding ability is beneficial or not for
30 achieving an optimal lyse and an optimal therapeutic effect.

Immune therapy experiments with SEB chemically conjugated to a tumor specific anti-idiotypic antibody have previously been described by Ochi et al., (J. Immunol. 151 (1993) 3180-3186).

During the prosecution of the priority application the Swedish
35 Patent Office has additionally cited Buelow et al. (J. Immunol. 148 (1992) 1-6) that describes fusions between Protein A and

fragments of SEB without emphasis of the MHC classes II binding or use of the fusion for cell killing; and Hartwig et al. (Int. Immunol. 5 (1993) 869-875) that describes mutations affecting MHC class II binding of the non-fused form of the superantigen streptococcal erythrogenic toxin A.

The objectives of the invention

A first objective of the invention is to improve previously known superantigen-antibody conjugates with respect to general immune stimulation versus directed cytotoxicity. Stimulation results in activated T-lymphocytes and is dependent on the ability of the superantigen to bind to both the T cell receptor and an MHC class II antigen.

A second objective of the invention is to provide conjugates between biospecific affinity counterparts (e.g. antibodies) and superantigens with a modified affinity for MHC class II antigens. This has now been shown to improve the selectivity for superantigen antibody dependent cell cytolysis (SADCC) of cells exposing the antigen (against which the antibody/biospecific affinity counterpart of the conjugate is directed) over other cells exposing MHC class II antigens.

A third objective of the invention is to provide conjugates that can be used as the active principle in the treatment of mammals suffering from cancers, autoimmune diseases, parasitic infestations, viral infections or other diseases associated with cells that on their surface express structures that are specific for respective disease.

The invention

- The main aspect of the invention is a conjugate comprising
- a. a biospecific affinity counterpart that is directed towards a structure to which one intends to bind to the conjugate,
 - b. a peptide that
 - i. is derived from a superantigen,
 - ii. has the ability to bind to the V β chain of the T cell receptor, and

iii. has a modified ability to bind to MHC class II antigens compared to the superantigen from which the peptide is derived (wild-type of superantigen = SA(wt)).

5 The peptide and the affinity counterpart are covalently linked to each other via a bridge (B).

The preferred conjugates have the ability to activate and direct T-lymphocytes to selective lysis of cells that on their surface expose the structure against which the affinity
10 counterpart is directed. This means that the conjugates shall cause cytolysis in an SADCC mediated method (Superantigen Antibody Dependent Cellular Cytotoxicity). See the experimental part below and our previous publications concerning conjugates between superantigens and antibodies (e.g. Dohlsten et al., WO
15 9201470).

The inventive conjugates have a structure that is analogous to the superantigen-antibody conjugates described in the prior art (Dohlsten et al., WO 9201470 which hereby is incorporated by reference), i.e. the conjugates complies with the formula:

20
$$T-B-SA(m)$$

where T represents the biospecific affinity counterpart, SA(m) is the modified superantigen (the above-mentioned peptide), and B is a covalent bridge linking T and SA(m) together.

T can in principle be any structure that binds via biospecific
25 affinity. In most important cases, T is capable of binding to a cell surface structure, preferably a disease specific structure as given above. The structure against which T is directed is usually different from (a) the V β chain epitope to which the superantigen derived peptide (SA(m)) binds and (b) the MHC class
30 II antigen epitope to which the unmodified superantigen binds. The biospecific affinity counterpart T may primarily be selected among interleukins (e.g. interleukin-2), hormones, antibodies and antigen binding fragments of antibodies, growth factors etc. See for instance Woodworth, Preclinical and Clinical Development of
35 Cytokine Toxins presented at the conference "Molecular Approaches to cancer Immunotherapy", Ashville, North Carolina, November 7-

11, 1993. Polypeptides binding to the constant domains of immunoglobulins (e.g. Proteins A and G and L), lectins, streptavidin, biotin etc were at the priority date considered to be of minor importance.

5 At the priority date, it was preferred that T was an antibody or an antigen binding fragment of an antibody (including Fab, F(ab)₂, Fv, single chain antibody etc), with particular emphasis of an antibody active fragment (such as Fab) of antibodies directed against the so called C242 epitope (Lindholm et al., WO
10 9301303) or against other cancer specific epitopes.

In case T is an antibody it is primarily monoclonal or a mixture of a defined number of monoclonals (e.g. 2, 3, 4, 5 or more). T may be a polyclonal antibody, in case the use is non-therapeutical.

15 It is not imperative for T to have a polypeptide structure.

The modified superantigen SA(m) is primarily a mutated superantigen but may potentially also be a chemically modified superantigen, including fragments of superantigens retaining the ability to bind to the V β chain of the T cell receptor.

20 The expression "mutated superantigen" means that the native ability of the superantigen to bind to MHC class II antigens has been modified on the genomic level by replacing, inserting or removing one or more amino acids in the native superantigen.

Superantigen fragments obtained by mutations removing parts of
25 the full amino acid sequence and fragments obtained by enzymatic or chemical cleavage of superantigens may be used equivalently in chemical conjugates of the invention.

The modified superantigen SA(m) may comprise one or more amino acid sequences that are derived from different superantigens and
30 that may have been mutated, for instance combinations of the preferred superantigens mentioned below.

The modified superantigen SA(m) as such may exhibit a decreased immunogenicity and toxicity compared to the native superantigen.

35 Other groups/substances that are capable of cross reacting with the V β -chain of the T cell receptor may potentially also be

employed equivalently with the mutated superantigen (SA(m)) as given above. Such groups/substances may be of non-polypeptide structure.

At the end of the priority year the most interesting product
5 candidates of the invention comprised mutated forms of
superantigens having multiple MHC class II binding sites and/or
the ability to coordinate Zn^{2+} , for instance SEA, SED, SEE and
SEH.

T as well as SA(m) may be prepared by recombinant techniques.

10 The bridge B may be selected as previously described (Dohlsten
et al., WO 9201470), i.e. it shall preferably be hydrophilic and
exhibit one or more structure(s) selected among amide, thioether,
ether, disulfide etc. In case the bridge have unsubstituted
unbroken hydrocarbon chains they preferably lack aromatic rings,
15 such as phenyl. The most important bridges are those obtained by
recombinant techniques, i.e. when the conjugation takes places on
the genomic level. In such cases oligopeptide bridges containing
hydrophilic amino acid residues, such as Gln, Ser, Gly, Glu and
Arg, are preferred. Pro and His may also be included. During the
20 priority year it has been decided that the preferred bridge is a
peptide comprising three amino acid residues (GlyGlyPro).

The inventive conjugate may comprise one or more modified
superantigen(s) per biospecific affinity counterpart and vice
versa. This means that T in the formula above may contain one or
25 more modified superantigens in addition to the biospecific
counterpart. In analogy SA(m) may contain one or more biospecific
affinity counterpart(s) T. The affinity counterpart T and SA(m)
may also comprise other structures. The number of modified
superantigens per affinity counterpart is preferably one or two.

30 The synthesis of the novel inventive conjugates may be carried
out in principle according to two main routes: 1. by recombinant
techniques and 2. chemical linking of T to SA(m). The methods are
well recognized for the ordinary skilled worker in the field and
comprise a large number of variants. It follows that the
35 invention primarily concerns artificial conjugates, i.e.
conjugates that are not found in nature.

Chemical linking of a modified superantigen to the biospecific affinity counterpart T often utilizes functional groups (e.g. primary amino groups or carboxy groups) that are present at many positions in each compound. It follows that the final product will contain a mixture of conjugate molecules differing with respect to the position at which linking has taken place.

For recombinant conjugates (fusion proteins) the obtained conjugate substance will be uniform with respect to the linking position. Either the amino terminal of the modified superantigen is linked to the carboxy terminal of the biospecific affinity counterpart or vice versa. For antibodies, such as intact antibodies and antigen binding fragments (Fab, Fv etc), either the light or the heavy chain may be utilized for such fusions. At present time recombinant conjugates are preferred, with preference for Fab fragments and linking of the amino terminal of the modified superantigen to the first constant domain of the heavy antibody chain (CH1), without exclusion of the analogous linking to the light chain or to the VH and VL domain that also may give quite good results.

There are two different methods for obtaining large amounts of superantigens (including modified and fused forms) in E. coli: intracellular production or secretion. The latter method is preferred for the inventive conjugates because it offers purification of correctly folded protein from the periplasma and from the culture medium. Intracellular production results in a complicated purification procedure and often needs refolding in vitro of the protein (in order for the protein to obtain the correct tertiary structure). The above does not exclude that it is possible to produce active conjugates also in other host cells, e.g. eukaryotic cells, such as yeast or mammalian cells.

The production of mutated superantigens and selection of mutants having a modified ability to bind (affinity) to MHC class II antigens may be carried out according to known techniques (see e.g. Kappler et al., J. Exp. Med. 165 (1992) 387-396). See also our experimental part.

The ability of the conjugate to bind to the T cell receptor $V\beta$ chain, to the target structure and to cause lysis of the target cell depends on i.a. the peptide (SA(m)) that is derived from a superantigen, the biospecific affinity counterpart (T) and the structure and length of the bridge (B). A person ordinary skilled in the art is able to optimize the inventive conjugates with respect to the binding ability and the ability to cause lysis by studying the relationship between effect and structure with the aid of those models that have been disclosed in connection with previously known superantigen antibody conjugates (see the above-referred publications). See also the experimental part below.

By modified ability to bind MHC class II antigens is primarily intended that the ratio $IC_{50}(SA(wt)):IC_{50}(SA(m))$ is < 0.9 (90 %), such as < 0.5 (< 50 %) and possibly also < 0.01 (< 1 %). In the alternative the modified binding ability of the inventive conjugates can be measured as the ratio of the dissociation constants $K_d(SA(wt)):K_d(SA(m))$ with K_d measured in nM and with the same limits as for the ratio $IC_{50}(SA(wt)):IC_{50}(SA(m))$. For the determination of $IC_{50}(SA(wt))$, $IC_{50}(SA(m))$, $K_d(SA(m))$ and $K_d(SA(wt))$ see the experimental part below.

It is previously known that certain superantigens may have two or more sites that bind to MHC class II antigen (Fraser et al., In: Superantigens: A pathogens view on the immune system. Eds. Huber & Palmer, Current Communications in Cell Molecular Biology 7 (1993) 7-29). For this type of superantigens the binding ability shall be modified at least one of the binding sites, e.g. as a reduction of the above-mentioned size. Possibly it may suffice with a superantigen modification that create a changed difference in affinity for two MHC class II binding sites, tentatively > 10 % and preferably by reducing the affinity of at least one site.

Superantigens bind to TCR $V\beta$ chains of different subgroups with varying affinities. In the inventive fusion proteins/conjugates, the superantigen employed may have been modified so as to show an altered subgroup specificity or an altered affinity to one or more members of the subgroup. There

are strong reasons to believe that a parabolic relationship exists between the affinity for TCR V β and stimulation via TCR, i.e. a moderate affinity will give the maximal stimulation. Accordingly an appropriate affinity of a modified superantigen for TCR V β may be at hand as soon as the fusion protein/conjugate comprising the modified superantigen is able to significantly stimulate a resting T cell population representing essentially the distribution of all human V β subgroups to proliferate. The T cell population may be pooled T cells from randomly selected human individuals. By significantly is meant that the stimulation is possible to measure. The results presented in Table II (right column) in the experimental part indicate that the ability to cause SADCC of the inventive conjugates/fusion proteins often is essentially the same as for the fusion comprising the wild-type superantigen.

Main use of the conjugates/fusion proteins of the invention.

The conjugates according to the invention are primarily intended for the treatment of the same diseases as the conjugates between normal superantigens and antibodies. See the above-mentioned publications. Thus the inventive conjugates may be administered either as the main therapy or as adjuvant therapy in connection with surgery or other drugs.

The pharmaceutical composition of the invention comprises formulations that as such are known within the field but now containing our novel conjugate. Thus the compositions may be in the form of a lyophilized particulate material, a sterile or aseptically produced solution, a tablet, an ampoule etc. Vehicles such as water (preferably buffered to a physiologically pH value by for instance PBS) or other inert solid or liquid material may be present. In general terms the compositions are prepared by the conjugate being mixed with, dissolved in, bound to, or otherwise combined with one or more water-soluble or water-insoluble aqueous or non-aqueous vehicles, if necessary together with suitable additives and adjuvants. It is imperative that the vehicles and conditions shall not adversely affect the activity

of the conjugate. Water as such is comprised within the expression vehicles.

Normally the conjugates will be sold and administered in predispensed dosages, each one containing an effective amount of the conjugate that, based on the result now presented, is believed to be within the range of 10 μ g - 50 mg. The exact dosage varies from case to case and depends on the patient's weight and age, administration route, type of disease, antibody, superantigen, linkage (-B-) et.

The administration routes are those commonly known within the field, i.e. a target cell lysing effective amount or a therapeutically effective amount of a conjugate according to the invention is contacted with the target cells. For the indications specified above this mostly means parenteral administration, such as injection or infusion (subcutaneously, intravenously, intra-arterial, intramuscularly) to a mammal, such as a human being. The conjugate may be administered locally or systemically.

By "target cell lysing effective amount" is contemplated that the amount is effective in activating and directing T-lymphocytes to destroy the target cell.

At the end of the priority year it had been decided that the preferred administration route for conjugates/fusion proteins comprising unmodified superantigens is 3 hours' intravenous infusion per day combined with a fever-reducing agent (paracetamol). The administration is to be repeated during 4 days and stopped before dsecondary antibodies are raised against the fusion protein/conjugate in the patient. This dosage schedule is likely to be applicabple also to the present inventive conjugates/fusion proteins.

Alternative fields of use.

The inventive conjugates can also be employed to quantitatively or qualitatively detect the structure against which the target-seeking group (T) is directed. In general these methods are well-known to people in the field. Thus, the modified superantigen may function as a marker group within immunoassays

including immunohistochemistry meaning that the marker group in turn is detected by for instance an antibody that is directed towards the peptide (SA(m)) and labelled with an enzyme, isotope, fluorophor or some other marker group known per se. Another
5 immunoassay method is to detect in a cell population cells that on their surface express a structure capable of binding to the target-seeking group (T). This use means that a sample from the cell population is incubated with T-lymphocytes together with the present inventive conjugate as in an SADCC assay. In case the
10 incubation leads to cell lysis this is an indication that the population contains cells that on their surface express the structure.

EXPERIMENTAL PART

15 MANUFACTURE OF RECOMBINANT PROTEINS

Antibodies

The experimental work in connection with the invention has primarily been done with monoclonal antibody C215 as a model substance. This antibody is directed against an antigen in the
20 GA-733 family (see for instance EP 376,746) and references cited therein and Larsson et al., Int. J. Canc. 32 (1988) 877-82). The C215 epitope has been judged not to be sufficiently specific for cancer treatment in humans. At the priority date mab C242 (Lindholm et al., WO 9301303) was believed to be a better
25 candidate, as judged from experiments with its fusion product with wild-type SEA.

Bacterial strains and plasmids

The E. coli strains UL635 (xyl-7, ara-14, T4^R, ΔompT) and HB101
30 (Boyer and Roulland-Dessoix, J. Mol. Biol. 41 (1969) 459-472) were used for the expression and cloning, respectively. The vector pKP889 was used for expression of Fab-SEA fusion proteins (derived from the murine antibody C215) and the vectors pKP943 and pKP1055 for secretion of SEA (Fig 1). The Fab-SEA expression
35 vector pKP889 is identical to pKP865 (Dohlsten et al, Proc. Natl. Acad. Sci. USA (1994) in press) except that the spacer between

12 SEQ. ID NO. 1

13 CH1 and SEA is GlyGlyAlaAlaHisTyrGly_A. Expression from pKP943 yields SEA with the native amino terminus. The use of pKP1055 results in SEA having a Gly residue added at the amino terminus. In both vectors the signals from staphylococcal protein A (Uhlén et al., J. Biol. Chem. 259 (1984) 1695-1702) are used for transcription and translation and a synthetic signal peptide for secretion (L. Abrahmsén, unpublished).

In vitro mutagenesis

10 Mutations were made by polymerase chain reactions run on a Perkin Elmer Thermocycler. The reaction mixture (100 µl) contained: 1 x PCR buffer from Perkin Elmer Cetus (10 mM Tris/HCl pH 8.3, 1.5 mM MgCl₂, 0.001 % (w/v) gelatine, an additional 2 mM MgCl₂, 0.4 mM dNTPs (Perkin Elmer Cetus), 2.5 units of Ampli Taq DNA polymerase (Perkin Elmer Cetus, USA) and 100 ng DNA template. Primers were added to a final concentration of 0.8 µM. The original template was a plasmid containing Staphylococcus aureus enterotoxin A gene identical to the one published by Betley et al. (J. Bacteriol. 170 (1988) 34-41), except that the first codon (encoding Ser) was changed to TCC to furnish a Bam HI site at the 5' end of the gene. Later a derivative containing more unique restriction enzyme sites introduced by silent mutations was used. Mutations introduced next to a restriction site were made with one set of primers, one of these spanning the mutation and the restriction site. For most mutations two set of primers had to be used and the PCR was performed in two consecutive steps. A new restriction enzyme site was introduced together with each mutation to enable facile identification. Oligonucleotides used as primers were synthesized on a Gene Assembler (Pharmacia Biotech AB, Sweden). To confirm each mutation the relevant portion of the nucleotide sequence was determined on an Applied Biosystems DNA-Sequenser using their Taq DyeDeoxy Termination Cycle Sequencing Kit.

Protein production and analysis

E. coli cells harboring the different gene constructs were grown overnight at room temperature (Fab-SEA vectors) and at 24-34°C (secretion vectors, the optimum depends on the mutation).

- 5 The broth was 2 x YT (16 g/l Bacto trypton, 10 g/l Bacto yeast extract, 5 g/l NaCl) supplemented with kanamycin (50 mg/l). Fusion proteins were induced by addition of isopropyl- β -D-thiogalactoside to a final concentration of 100 μ M. (The protein A promotor used in the expression of non-fused SEA is
- 10 constitutive). The cells were pelleted at 5000 x g and the periplasmic contents were released by gently thawing the previously frozen cell pellet in 10 mM Tris-HCl (pH 7.5) on ice during agitation for 1 hour. The periplasmic extracts were clarified by centrifugation at 9500 x g for 15 minutes. The Fab-
- 15 SEA proteins were used without further purification. SEA and Gly-SEA were further purified by affinity chromatography on an anti-SEA antibody column. Polyclonal rabbit anti-SEA antibodies were previously collected from rabbits preimmunized with SEA and purified by affinity chromatography on protein G Sepharose®
- 20 (Pharmacia Biotech).

Protein Analysis

- The proteins were separated in precast polyacrylamide SDS Tris-Glycine Novex gels (gradient 4-20 % or homogenous 12 %,
- 25 Novex novel experimental technology) and either stained with Coomassie Blue or used in Western blot. Polyclonal rabbit anti-SEA antibodies (above) were used to detect SEA in Western blot analysis, followed by porcine anti-rabbit Ig antibodies, and rabbit anti-horseradish peroxidase antibodies and peroxidase.
- 30 With Fab-SEA fusion proteins peroxidase conjugated rat antibodies recognizing the kappa chain were also used (AAC 08P, Serotech LTD, England). 3,3'-diaminobenzidine (Sigma) was used for visualization of peroxidase.

- Circular dichroism (CD) spectra were collected in a J-720
- 35 spectropolarimeter (JASCO, Japan) at room temperature (22-25°C) in 10 mM phosphate buffer, pH 8.2, with 0.02 mM ZnSO₄ and 0.005 %

(v/v) Tween® 20. The scanning speed was 10 nm/min and each spectrum was averaged from five subsequent scans. The cell path length was 1 mm and the protein concentration 0.2 to 0.5 mg/ml. Guanidine hydrochloride (Gdn-HCl) denaturations at equilibrium were measured at 23°C by CD at 222 nm with a protein concentration of 0.3 mg/ml and a cell path length of 1 mm. These data were used to calculate the apparent fraction of unfolded protein (F_{app}). Equilibrium unfolding parameters were derived by fitting the data to a two-site folding process (Hurle et al., Biochemistry 29 (1990) 4410-4419).

BINDING AND FUNCTIONAL ASSAYS IN VITRO

Materials

Reagents: RPMI 1640 medium obtained from Gibco, Middlesex, England was used. The medium had a pH of 7.4 and contained 2 mM L-glutamine (Gibco, Middlesex, England), 0.01 M HEPES (Biological Industries, Israel), 1 mM NaHCO₃ (Biochrom AG, Germany), 0.1 mg/ml Gentamycin sulphate (Biological Industries, Israel), 1 mM Na-pyruvate (JRH Biosciences Industries, USA), 0.05 mM mercaptoethanol (Sigma Co., USA), 100 times concentrated non-essential amino acids (Flow Laboratories, Scotland) and was supplemented with 10 % fetal bovine serum (Gibco, Middlesex, England). Recombinant SEA(wt), SEA(m) and the fusion products C215Fab-SEA(wt) and C215Fab-SEA(m) were obtained as described above. Human recombinant IL-2 was from Cetus Corp., USA. Mitomycin C was from Sigma Co., USA. Na₂⁵¹CrO₄ was obtained from Merck, Germany. Phosphate buffered saline (PBS) without magnesium and calcium was received from Imperial, England.

Cells: The human colon carcinoma cell line Colo205 and the B cell lymphoma cell line Raji were obtained from American Type Cell Culture Collection (Rockville, MD, USA) (expressing HLA-DR3/w10, -DP7, -DQw1/w2). The EBV-transformed lymphoblastoid B cell line BSM was a generous gift from Dr van De Griend, Dept of Immunology, Dr Daniel den Hoed Cancer Center, Leiden, the Netherlands. The cells were repeatedly tested for mycoplasma

contamination with Gen-Probe Mycoplasma T.C. test, Gen-Probe Inc., San Diego, USA.

SEA activated T cell lines were produced by activation of mononuclear cells from peripheral blood. The blood was received as buffy coats from blood donors at the University Hospital of Lund. The PBMs were stimulated at a concentration of 2×10^6 cells/ml with mitomycin C treated SEA coated BSM cells (preincubated with 100 ng/ml SEA) in medium with 10% FCS. The T cell lines were restimulated biweekly with 20 U/ml human recombinant IL-2 and weekly with mitomycin C treated SEA coated BSM cells. The cell lines were cultivated for 4-12 weeks before being used in the assay.

The viability of the effector cells, as determined by trypan blue exclusion, exceeded 50 %.

Determination of MHC class II binding characteristics of wild-type and mutant SEA

Radioiodination procedure. Appropriate amounts of wild-type or mutant SEA were radiolabeled with 10 to 25 mCi Na^{125}I using enzymobeads with the lactoperoxidase technique (NEN, Boston, MA). The reaction was stopped by quenching with sodium azide and protein-bound radioactivity was separated from free iodine by filtration through a PD-10 column (Pharmacia Biotech AB, Sweden) with R10 medium as elution buffer. Conditions were chosen to obtain a stoichiometric ratio between iodine-125 and protein of $\leq 2:1$. The radiochemical purity was verified by size-exclusion chromatography on a TSK SW 3000 HPLC column. The effect of the radioiodination on the binding activity was only tested for wild-type SEA and found not to be affected (data not shown).

Direct binding assay. Raji cells, $6 \times 10^4/100 \mu\text{l}$, previously cultivated in R10 medium, were added to conical polypropylene tubes and incubated ($22^\circ\text{C}/45 \text{ min}$) in triplicate with 100 $\mu\text{l}/\text{tube}$ of serially diluted ^{125}I -labeled wild-type or mutant SEA. The cells were washed with 2 ml 1 % (w/v) bovine serum albumin (BSA) in 10 mM phosphate-buffered saline (PBS), pH 7.4, centrifugated at $300 \times g$ for 5 minutes and aspirated. This

procedure was repeated twice. Finally, the cells were analyzed for cell-bound radioactivity in a gamma counter (Packard Instruments Co, Downers Grove, IL, USA). The apparent dissociation constant, K_d , and the number of binding sites, N , at saturation were calculated according to Scatchard (Ann. N.Y. Acad. Sci. 51 (1949) 660-72) after subtraction of non-specific binding (i.e. binding after incubation with R10 medium alone.

Inhibition assay (inhibition of ^{125}I -labeled wild-type SEA binding by mutant SEAs). These inhibition experiments were carried out as is described for the direct binding assay with slight modifications. Briefly, 50 μl of ^{125}I -labeled wild-type SEA was allowed to compete with an excess of unlabeled wild-type or mutant SEA (50 μl /tube) for binding to 6×10^4 /100 μl Raji cells. A tracer concentration yielding $\approx 40\%$ bound radioactivity in the direct assay was used to obtain maximal sensitivity in the inhibition assay. The displacement capacity of the competitor was expressed as the concentration yielding 50% inhibition (IC_{50}) of bound radioactivity. The binding affinity of the mutants relative to wild-type SEA was calculated using the equation:

$$\text{IC}_{50}(\text{SEA}(\text{wt})) : \text{IC}_{50}(\text{SEA}(\text{m}))$$

In order to analyze whether the mutants compete for binding to the same site on Raji cells as wild-type SEA, the binding data obtained with SEA mutants were plotted as a log-logit function and tested for parallelism with the corresponding data for wild-type SEA.

Inhibition assay (inhibition of the binding of fluorescent-labeled wild-type SEA by unlabeled wild-type SEA and SEA mutants). Raji cells (2.5×10^5) were incubated with inhibitor (wild-type or mutant SEA; 0-6000 nM) diluted in 50 μl CO_2 -independent medium (Gibco) supplemented with 10% FCS, glutamine and gentamycin at 37°C for 30 minutes. Fluorescein conjugated wild-type SEA was added to a final concentration of 30 nM and the samples were incubated for an additional half hour at 37°C . The samples were washed three times with ice cold PBS supplemented with 1% BSA (PBS-BSA) and finally kept in 0.4 ml PBS-BSA on ice until they were analyzed. From each sample 10 000 live cells were

analyzed for green fluorescence on a FACStar® (Becton Dickinson) flow cytometer and the mean fluorescence value was calculated using the LYSIS II program.

5 **SDCC and SADCC assays of SEA(wt), SEA(m) and their fusion proteins with C215Fab.**

SDCC-assays. The cytotoxicity of SEA(wt), SEA(m) and their fusions with C215Fab against MHC class II⁺ Raji cells was analyzed in a standard 4 hour ⁵¹Cr³⁺-release assay, using in
10 vitro stimulated SEA specific T cell lines as effector cells. Briefly, ⁵¹Cr labeled Raji cells were incubated at 2.5×10^3 cells per 0.2 ml medium (RPMI, 10 % FCS) in microtitre wells at defined effector to target cell ratio in the presence or absence (control) of the additives. Percent specific cytotoxicity was
15 calculated as $100 \times ([\text{cpm experimental release} - \text{cpm background release}] / [\text{cpm total release} - \text{cpm background release}])$. The effector to target cell ratio was 30:1 for unfused SEAs and 40:1 for fusion proteins.

SADCC against of human colon cancer cells. The cytotoxicity of
20 C215Fab-SEA(wt), C215Fab-SEA(m), SEA(wt) and SEA mutants against C215⁺ MHC class II⁻ colon carcinoma cells SW 620 was analyzed in a standard 4 hour ⁵¹Cr³⁺-release assay, using in vitro stimulated SEA specific T cell lines as effector cells. Briefly, ⁵¹Cr³⁺-labeled SW 620 cells were incubated at 2.5×10^3 cells per 0.2 ml
25 medium (RPMI, 10 % FCS) in microtitre wells at effector to target cell ratio 30:1 in the presence or absence (control) of the additives. Percent specific cytotoxicity was calculated as for SDCC assays.

30 **IN VIVO FUNCTIONAL EXPERIMENTS**

Tumor cells. B16-F10 melanoma cells transfected with a cDNA encoding the human tumor associated antigen C215 (B16-C215) (Dohlsten et al., Monoclonal antibody-superantigen fusion proteins: Tumor specific agents for T cell based tumor therapy;
35 Proc. Natl. Acad. Sci. USA, In press, 1994), were grown as adherent cells to subconfluency. The culture medium consisted of

RPMI 1640 (GIBCO, Middlesex, UK) supplemented with 5×10^{-5} β -mercaptoethanol (Sigma, St Louis, MO, USA), 2 mM L-glutamine (GIBCO), 0.01 M Hepes (Biological Industries, Israel) and 10 % fetal calf serum (GIBCO). The cells were detached by a brief incubation in 0.02 % EDTA and suspended in ice cold phosphate buffered saline with 1 % syngeneic mouse serum (vehicle) to 4×10^5 cells/ml.

Animals and animal treatment. The mice were 12-19 weeks old C57Bl/6 mice transgenic for a T cell receptor V β 3 chain (Dohlsten et al., Immunology 79 (1993) 520-527). One hundred thousand B16-C215 tumor cells were injected i.v. in the tail vein in 0.2 ml vehicle. On day 1, 2 and 3, the mice were given i.v. injections of C215Fab-SEA(wt) or C215Fab-SEA(D227A) in 0.2 ml vehicle at doses indicated in the figures 5a and 5b. Control mice were given only vehicle according to the same schedule. On day 21 after tumor cell injection, the mice were killed by cervical dislocation, the lungs removed, fixed in Bouin's solution and the number of lung metastases counted.

20 RESULTS

"Alanine scanning" of staphylococcal enterotoxin A.

Initially the structure of SEA was unknown and only speculations could be done about what side chains were surface accessible. Therefore, the majority of the mutants were chosen from alignments of homologous superantigens (Marrack and Kappler, Science 248 (1990) 705-711). Conserved (mainly polar) residues were chosen on the rational that some of these superantigens are expected to bind to HLA-DR in a conserved fashion (Chitagumpala et al., J. Immunol. 147 (1991) 3876-3881). Alanine replacements were used according to published strategies (Cunningham and Wells, Science 244 (1988) 1081-1085). During the course of this work the available information increased: i) it was shown that a Zn^{2+} ion is important for the interaction between SEA and MHC class II (HLA-DR) (Fraser et al., Proc. Natl. Acad. Sci. USA 89 (1991) 5507-5511), ii) a mutational analysis of staphylococcal enterotoxin B (SEB) was presented (Kappler et al., J. Exp. Med.

175 (1992) 387-396), and iii) the structure of SEB was presented (Swaminathan et al., Nature 359 (1992) 801-806).

Our first mutant showing a severely reduced affinity for HLA-DR, D227A, was found to co-ordinate the Zn^{2+} ion very poorly (data not shown). Assuming a common fold for SEA and SEB, the new data suggested two MHC class II binding regions; one involving the Zn^{2+} ion and one corresponding to the site defined in SEB. A second set of mutations were made on these assumptions. This second set of mutants were expressed in the form of SEA carrying a glycine added at the amino terminus. First the extension was shown to have no effects on the binding properties of wild-type SEA (next section).

Most of the mutants were expressed and secreted by E. coli in a functional form as judged by analysis of the binding of monoclonal antibodies (Table I). Very low amounts were obtained of the mutants E154A/D156A and R160A. Consequently these were excluded from the study. The mutants having an Ala substitution in residues 128, 187, 225 or 227 were not recognized by the monoclonal antibody 1E. The latter two mutants showed a reduced level of expression (more pronounced at 34°C than at 24°C) and migrated faster during SDS-PAGE, under denaturing but not reducing conditions (all other mutants migrated as wild-type SEA, data not shown). As judged by CD spectra analysis the structure of D227A could differ slightly from native SEA (figure 2), but the stability was very close to wild-type SEA (measured as resistance towards guanidine hydrochloride denaturation). The calculated $\Delta\Delta G$ between the mutant and native SEA (SEA(wt)) was - 0.16 kcal/mol and is only about 4 % of the ΔG° values (data not shown). Overall the signals in the CD analysis were low, as expected from a mostly β -sheet structure. It was recently reported that His 225 co-ordinates Zn^{2+} (unpublished data in Fraser et al (Proc. Natl. Acad. Sci. USA 89 (1991) 5507-5511). Since Asp 227 is involved in Zn^{2+} co-ordination (above) and presumably located in the same β -sheet as His 225 this suggests that these two residues constitutes the zinc-binding nucleus

found in zinc-co-ordinating proteins (Vallee and Auld, Biochemistry 29 (1990) 5647-5659).

Binding to MHC class II and T cell receptor

5 The MHC class II affinity was calculated from the amounts needed to compete with fluorescein-labeled wild-type SEA for Raji cell exposing large amounts of MHC class II. The displacement capacity of a mutant was calculated from the concentration yielding 50 % inhibition (IC_{50}) of bound fluorescence compared
10 with the concentration needed with wild-type SEA as the competitor. For wild-type SEA and for some mutants, the result from this analysis was compared with the result from an analysis where ^{125}I labeled wild-type SEA was used as the tracer. As may be seen in Table II, the values obtained from these two
15 inhibition analyses correlate well

For six selected mutants the binding to MHC class II was measured directly using ^{125}I labeled mutant SEA (Table II). With the mutant H50A the values obtained from the direct binding assay and the inhibition assays correlated well but with the mutant
20 F47A a large discrepancy was found: the direct binding indicated only 7 times weaker binding than wild-type SEA but both competition analyses demonstrated around 70 times reduced binding. The data from two of the other mutants indicated two separate binding interactions. For the mutants H225A and D227A
25 the affinity was below the detection limit also in this analysis.

We previously showed that fusion proteins composed of the Fab fragment of a carcinoma reactive antibody and SEA could be used to direct cytotoxic T cells to specifically lyse cancer cells, while the interaction between SEA and the T cell receptor (TCR)
30 was too weak to be detected by itself (Dohlsten et al., Proc. Natl. Acad. Sci. USA, in press). Thus, in contrast to analyses involving the isolated superantigen the Fab fusion context enables a functional assay for the interaction between SEA and the TCR, independent of the MHC class II binding. Consequently,
35 the efficiency of the different conjugates to direct T cells to lyse cells recognized by the Fab moiety was monitored in a

chromium release assay. This analysis confirmed that the mutations shown to affect the MHC class II binding did not affect the TCR binding (Table II).

5 Biological effects of the mutations

The proliferative effect was measured as the ability to stimulate peripheral lymphocytes to divide. All three mutants that competes very poorly for MHC class II induced little or no proliferation and the intermediate mutant H187A displayed some proliferative capacity, whereas the other investigated mutants were indistinguishable from the wild-type (table III). Harris et al (Infect. Immun. 61 (1993) 3175-3183) recently reported a similar severe reduction in T cell stimulatory activity for the SEA mutants F47G and L48G. Clearly a strong reduction in any of the two suggested binding regions results in a severe effect on the ability to induce proliferation. This suggests that SEA cross-links two molecules of MHC class II leading to dimerization of the TCR and that this is needed to yield a signal transduction.

In contrast the efficiency of the different mutants in directing in vitro stimulated SEA T cells to lyse MHC class II bearing target cells shows correlation with the binding affinity, rather than to the ability to compete (Table III). For example, the efficiency of F47A and D227A are only reduced 2.5 times and 300 times, respectively. Thus, here no inherent requirement for divalency too is obvious. The increase in multivalency resulting from the significantly larger number of TCRs on the surface of activated T cells might partially shield the effect of a lower avidity in the SEA/MHC class II interaction. That dimerization is not needed to direct T cell cytotoxicity has previously been demonstrated by the use of carcinoma specific bifunctional antibodies containing one anti-CD3 moiety and one anti-carcinoma moiety (Renner et al., Science 264 (1994) 833-35).

In vivo functional experiments: The results are represented in figures 6a and 6b. Treatment of mice with C215Fab-SEA(wt) and

C215Fab-SEA(D227A) were both highly effective in reducing the number of lung metastases of B16-C215 melanoma cells. The therapeutic effect was essentially identical for the two variants of the targeted superantigens. Treatment with C215Fab-SEA(wt) resulted in 70 % lethality at doses of 5 µg/injection. In contrast, no mice died when the same dose of C215Fab-SEA(D227A) were used. Taken together, SEA(D227A) is an example of a mutant with reduced toxicity and retained therapeutic efficiency when incorporated in a Fab-SEA fusion protein.

DISCUSSION

The structure of the complex between SEB and HLA-DR was recently reported (Jardetzky et al., Nature 368 (1994) 711-718). Most of the SEB residues identified to be involved in this interaction are conserved in SEA. Our data on mutant D227A indicates a weak affinity for the interaction between this site of SEA (the amino proximal site) and the MHC class II, having a K_d value higher than 8 µM. The K_d for the interaction between SEB and HLA-DR was recently reported to be 1.7 µM (Seth et al., Nature 369 (1994) 324-27). The different interactions between SEB, TCR and HLA-DR were investigated and it was shown that the complex between SEB and HLA-DR was not stably maintained in the absence of TCR. Plasmon resonance experiments indicated that this was because of a very fast off-rate. The avidity effects obtained if SEA cross-links two molecules of MHC class II followed by a subsequent dimerization of the TCR could explain how SEA may induce proliferative effects at concentrations well below the K_d . Assuming that the mutation F47A reduces the affinity of the amino proximal site below significance, the K_d of the Zn^{2+} site is around 95 nM. This hypothesis was recently strengthened by the observation that the mutants F47R, F47R/H50A and F47R/L48A/H50D show identical affinity for MHC class II as F47A (unpublished).

Based on the SEB structure (Kappler et al., J. Exp. Med. 175 (1992) 387-396) and on homology alignments (Marrack and Kappler, Science 248 (1990) 705-711), it is strongly suggested that His225 and Asp227 are located in the same β -sheet and thus the side

chains could be proximal. Thus, most likely these two residues constitute the zinc-binding nucleus found in zinc-co-ordinating proteins (Vallee and Auld, Biochemistry 29 (1990) 5647-5659). Similarly to these mutants, the mutants with a replacement at
5 residue 128 or 187 are also recognized by all monoclonals except 1E. Fraser et al (Proc. Natl. Acad. Sci. USA 89 (1991) 5507-5511) showed that Zn^{2+} is bound to SEA and is needed for a high affinity interaction with MHC class II. The affinity for zinc was not affected by the addition of HLA-DR. Based on this observation
10 and the high affinity for Zn^{2+} (K_d of around 1 μM) a co-ordination exclusively provided by SEA and involving 4 fold co-ordination was suggested. Our data indicates an involvement of the four residues N128, H187, H225 and D227. The function of the former two residues is not yet clear; instead of providing a
15 ligand N128 could help in the deprotonation of D227. One argument for this is that the effect of replacing D227 is more severe than when replacing H225.

It was previously reported that there is a lack of correlation between the affinity of different superantigens for the MHC class
20 II and the capacity to stimulate T cells to proliferate (Chintagumpala et al., J. Immunol. 147 (1991) 3876-3881). These results might partly be explained by different affinities of the superantigens towards different TCR V β -chains. Here we have observed the same lack of correlation but in contrast to separate
25 superantigens the mutants display identical TCR affinity as shown in the Fab-SEA context (measured as SADCC). The most likely explanation for the lack of correlation is that two binding regions identified in this analysis represent two separate binding sites that yields not only a co-operative binding, but
30 which results in the cross-linking of two molecules of MHC class II, which in turn yields dimerization of two molecules of the T cell receptor. This would imply that the affinity of both sites are important to obtain the proliferative effect. A high avidity results from the interactions within a hexameric complex
35 involving two molecules of SEA, TCR and MHC class II. Thus the

strong affinity/avidity of SEA towards MHC class II enables SEA interaction with the TCR despite a low direct affinity.

Other biospecific affinity counterparts: A fusion protein of

5 SEA(D227A) and an IgG-binding domain of staphylococcal protein A has been produced by recombinant technology and expressed in E. coli. This reagent has successfully been used to target T-lymphocytes to Mot 4 and CCRF-CEM cells (obtained from ATCC) that are CD7 and CD38 positive but HLA-DP, -DQ and -DR negative. The
10 Mot 4 and CCRF-CEM cells were preincubated with anti-CD7 or anti-CD38 mouse monoclonals (Dianova, Hamburg, Germany). In order to enhance binding between the mouse monoclonals and the IgG-binding part of the fusion protein rabbit anti-mouse Ig antibody was also added.

15 In comparison with protein A-SEA(wt), protein A-SEA(D227A) had a decreased ability to bind to Daudi cells expressing MHC class II antigen.

Table I

20 Confirmation of mutant structural integrity. The binding of six monoclonal antibodies was monitored.

	Mutation	Monoclonal antibody					
		1A	2A	3A	1E	4E	EC-A1
	Wild-type	+	+	+	+	+	+
25	D11A/K14A	+	+	+	+	+	+
	D45A	+	+	+	+	+	+
	F47A	+	+	+	+	+	+
	H50A	(+)	+	(+)	+	+	+
	K55A	+	+	+	+	+	+
30	H114A	+	+	+	+	+	+
	K123A/D132G	+	+	+	+	+	+
	N128A	+	+	+	-	+	+
	K147A/K148A	+	+	+	+	-	+
	E154A/D156A	ND	ND	ND	+	ND	ND
35	R160A	ND	ND	ND	+	ND	ND
	H187A	+	+	+	-	+	+

25

E191A/N195A	+	+	+	+	+	+
D197A	+	+	+	+	+	+
H225A	.	+	+	-	+	+
D227A	+	+	+	-	+	+

- 5 Footnotes: A plus sign indicates binding, parenthesis indicate 50 to 90 % binding compared with wild-type SEA. ND means not determined.

Table II

- 10 Binding of SEA mutants to the MHC class II and the T cell receptor. The latter was monitored as the ability to direct activated cytotoxic T-cells specifically to lyse carcinoma cells using Fab-SEA fusions of the different mutants (SADCC).

	Mutation	IC ₅₀ (nM) SEA-FITC ¹	IC ₅₀ (nM) ¹²⁵ I-SEA ¹	K _d (nM) ¹²⁵ I labeled ¹	SADCC(% of wild-type ¹
15	wild-type	50	38	13	100 ²
	Gly-SEA	50	ND	ND	100 ²
	D11A/K14A	50	ND	ND	ND
	D45A	53	ND	ND	ND
20	F47A	3150	2943	95	100
	H50A	150	132	32	100
	K55A	44	ND	ND	ND
	H114A	48	ND	ND	ND
	K123A/D132G	188	75	12/237	100
25	N128A	1150	ND	2.9/76	100
	K147A/K148A	58	ND	ND	ND
	H187A	1030	602	97	100
	E191A/N195A	51	ND	ND	ND
	D197A	78	ND	ND	ND
30	H225A	>9000	9600	ND	ND
	D227A	>9000	>10000	>8000	100

Footnotes: 1) ND means not determined. 2) In the Fab-SEA context the spacer between C_H1 and SEA ends with a Gly.

20520 569560

Table III

Biological effects of the mutations. The ability to stimulate resting T cells to proliferate and the ability to direct cytotoxic cells to lyse MHC class II exposing target cells were monitored (SDCC = Superantigen Dependent mediated Cellular Cytotoxicity).

	Mutation	Proliferation	SDCC
		%	EC ₅₀ (relative)
10	wild-type	100	1
	Gly-SEA	ND	1
	D11A/K14A	ND	0.8
	D45A	50	1.3
	F47A	<0.2	2.5
15	H50A	20	1.4
	K55A	100	1.3
	H114A	ND	1
	K123A/D132G	40	2.1
	N128A	40	1.2
20	K147A/K148A	ND	0.7
	E154A/D156A	ND	ND
	R160A	ND	ND
	H187A	15	4
	E191A/N195A	100	1.1
25	D197A	ND	1.3
	H225A	<0.2	3x10 ²
	D227A	<0.01	3x10 ²

Footnotes: ND means not determined.

30 ~~Legend to Table III~~
General: The mutant SEA(D227A) (=SEA(m9) or mutant m9) was at the priority date the most promising SEA variant. We have therefore selected to present in vitro and in vivo results with this variant (Figures 3-6).

Figure 1.

Schematic outline of the plasmids used to express SEA and C215Fab-SEA. The coding regions and the two transcription terminators following the product genes are indicated by boxes.

- 5 The gene encoding the kanamycin resistance protein is labeled Km. lacI is the lac repressor gene. V_H and C_H1 indicates the gene encoding the F_d fragment of the heavy chain of the murine antibody C215. Likewise V_K and C_K indicates the gene encoding the kappa chain. Rop is the gene encoding the replication control
10 protein from pBR322. The promoters directing transcription of product genes are shown as arrows, in pKP889 the trc promotor and in the other two vectors the promotor from staphylococcal protein A (spa). The region containing the origin of replication is indicated by ori. The only difference between SEA encoded by
15 pKP943 and pKP1055 is a glycine residue added at the N-terminus of the latter. The SEA gene contained in the latter vector also contains more unique restriction enzyme sites, introduced by silent mutations.

Figure 2

- 20 Circular dichroism spectra for wild-type SEA and for the mutants F47A and D227A, representing the most severely reduced mutations in each MHC class II binding region. The solid line is the curve for wild-type SEA. The curves for the mutants are dotted or center, F47A respectively D227A.

- 25 **Figure 3** shows the concentration dependency of superantigen dependent mediated cellular cytotoxicity (SDCC) for SEA(wt) and SEA(D227A).

- Figure 4** shows the concentration dependency of superantigen dependent cell mediated cytotoxicity (SDCC) for C215Fab-SEA(wt)
30 and C215Fab-SEA(D227A).

Figure 5 shows the concentration dependency of superantigen mAb dependent cell mediated cytotoxicity (SADCC) for C215Fab-SEA(wt) and C215Fab-SEA(D227A) compared to free SEA(wt).

- Figure 6a** compares the therapeutic effects obtained in C57Bl/6
35 mice carrying lung metastases of B16-C215 melanoma cells by treatment with C215Fab-SEA(wt) and C215Fab-SEA(D227A).

Figure 6b shows toxicity of C215-SEA(wt) and C215-SEA(D227A) for the treatments represented in figure 6a.

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